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Inhibition of avian leukosis virus replication by vector-based RNA interference

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Abstract

RNA interference (RNAi) has recently emerged as a promising antiviral technique in vertebrates. Although most studies have used exogenous short interfering RNAs (siRNAs) to inhibit viral replication, vectors expressing short hairpin RNAs (shRNA-mirs) in the context of a modified endogenous micro-RNA (miRNA) are more efficient and are practical for *in vivo* delivery. In this study, replication competent retroviral vectors were designed to deliver shRNA-mirs targeting subgroup B avian leukosis virus (ALV), the most effective of which reduced expression of protein targets by as much as 90% in cultured avian cells. Cells expressing shRNA-mirs targeting the *tvb* receptor sequence or the viral *env(B)* sequence significantly inhibited ALV(B) replication. This study demonstrates efficient antiviral RNAi in avian cells using shRNA-mirs expressed from pol II promoters, including an inducible promoter, allowing for the regulation of the antiviral effect by doxycycline.

Keywords: Avian leukosis virus; RNA interference; Micro-RNA; Replication-competent retroviral vector

Introduction

RNA interference (RNAi) is an evolutionarily conserved mechanism of gene silencing that has shown promise as an antiviral strategy in vertebrates (Cullen, 2006; Hu et al., 2002). RNAi is mediated by short RNA "guide strand" oligonucleotides that bind to, suppress translation of, and sometimes induce cleavage of complementary mRNAs. RNAi generally arises from two types of intermediary molecules: siRNAs and miRNAs (Tang, 2005; Valencia-Sanchez et al., 2006). siRNAs are ~22-bp double-stranded (ds) oligonucleotides that can be naturally processed from longer dsRNA but also can be chemically synthesized and introduced directly into cells or transcribed as short hairpin RNAs (shRNAs) that are then processed to siRNA (Nakahara and Carthew, 2004). Initially, shRNAs were most often transcribed from polymerase III (pol III) promoters delivered by exogenous DNA plasmids or retroviruses. More recently, endogenous miRNA genes, that are transcribed as larger RNA precursors from pol II promoters, have been found to more effectively generate an RNAi effect (Boden et al., 2004; Das et al., 2006; Sun et al., 2006). In this case, the stem region of a miRNA gene is replaced with the target sequence and its guide RNA complement (termed shRNA-mirs; Zeng et al., 2002; Silva et al., 2005; Dickins et al., 2005).

RNAi appears to play a large role in normal viral life cycles. Many viruses, including human immunodeficiency virus (HIV), herpesviruses and some adenoviruses, contain miRNAs as part of their genomes that may be used to suppress host genes or regulate their own gene expression (Cullen, 2006; Sullivan and Ganem, 2005). Also, RNAi has been shown to be an innate

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antiviral defense mechanism in plants, insects and nematodes, and recent evidence indicates that higher vertebrates, including humans, use miRNAs to protect against certain viral infections (Lecellier et al., 2005).

Experimental RNAi strategies have been used successfully to inhibit viral replication. Hu et al. (2002) showed that siRNAs containing sequences of the *gag* gene of avian leukosis virus (ALV), when electroporated into chicken embryos, were effective at slowing viral propagation. RNAi inhibition of HIV has been studied more extensively, and siRNAs against the host cell CD4 co-receptor, the HIV long terminal repeat (LTR), and the viral p24 structural protein, among others, have all been successful in reducing the growth of HIV (Martinez et al., 2002; Novina et al., 2002) . However, the transient nature of siRNAs and difficulties in delivery present major hurdles for *in vivo* application of this approach. RNAi delivered by DNA plasmids can be longer lasting but faces similar obstacles. Retroviral vectors provide the opportunity to stably integrate the RNAi cassette into the recipient cell genome, usually as a single copy provirus, and such vectors recently have been used in large scale RNAi mutagenesis of endogenous genes (reviewed in Chang et al., 2006; Root et al., 2006), but their use in inhibiting viruses has been limited.

This study describes a vector-based RNAi strategy against an important pathogen of chickens, ALV. ALV is an oncogenic retrovirus divided into different subgroups, designated A–J, based upon differences in the surface region (SU) of its envelope glycoprotein, encoded by the *env* gene (Coffin et al., 1997). Each viral subgroup varies in its prevalence and toxicity, with ALV(A), ALV(B), and ALV(J) cited as the most dangerous to agricultural chicken populations (Fadly and Smith, 1999). ALV enters a host cell through interactions between the viral envelope glycoprotein and a host cell receptor protein, which may differ between viral subgroups (Coffin et al., 1997). ALV (B) infection, the subgroup targeted in this study, is mediated through the host-cell receptor protein CAR-1, encoded by *tvb*, a member of the tumor necrosis factor receptor (TNFR) superfamily (Brojatsch et al., 1996, 2000). Here, we explore reducing expression of both a host receptor gene, *tvb*, and a viral gene, *env(B)*, to inhibit ALV replication. These two genes encode the proteins that mediate the virus–host cell interaction, the initial step in viral infection. We have modified a previously described replication-competent ALV(A) RNAi vector (Bromberg-White et al., 2004) to deliver gene-specific shRNA-mirs in the context of the endogenous chicken *miR-30a* gene. We demonstrate that our vector system shows potential as an antiviral RNAi agent in avian cells and is amenable to inducible promoter-driven expression.

Results

GFP fluorescence in DF-1 cells is decreased by RCASBP(A) shRNA-mirs

Retroviral entry vector plasmids were constructed containing 417 bp of the chicken *miR-30a* gene, slightly modified to provide useful restriction sites into which synthetic target sequence duplexes could be inserted (Materials and methods). Various entry vectors contained either no promoter or various pol III promoters (mouse U6, human H1, chicken U6-1, or chicken U6-2; Kudo and Sutou, 2005) with or without the 27 bp leader sequence normally present between U6 promoters and the start of U6 RNA (Paddison et al., 2004). Entry vectors with no promoter were recombined into a vector based on RCASBP (A) (*r*eplication *c*ompetent, *A*LV LTR promoter, *S*plice acceptor, *B*ryan-strain *pol* gene, sub-group *A*; Bromberg-White et al., 2004; Fig. 1). RCASBP vectors are designed such that the inserted sequence is expressed as a sub-genomic, spliced RNA transcribed from the viral LTR (Hughes et al., 1987). The shRNA-mir sequences downstream of pol III promoters were moved into the corresponding RCANBP(A) vector that lacks the relevant splice acceptor such that insert transcription depends on the internal promoter provided. Two target sequences within GFP were used (Materials and methods), along with a control sequence of the same length and similar base composition, but with a scrambled sequence. These viral vectors were propagated in DF-1 avian fibroblasts stably expressing GFP. Since these viruses are replication competent in avian cells, they quickly spread throughout culture thereby delivering the shRNA to the majority of the cells. Analysis of GFP expression by fluorescence-activated cell sorting (FACS) revealed that none of the RNA polymerase III promoter constructs reduced GFP expression. In addition, shRNA expression from the pol III constructs was not detectable by PAGE–Northern (data not shown). In contrast, both GFP target sequences achieved an 85–90% reduction in GFP fluorescence (Fig. 2) when transcribed from the viral LTR promoter in RCASBP(A), indicating a substantial and specific RNAi response.

ALV(B) resistance through silencing of the tvb host receptor

To reduce expression of the ALV(B) receptor, *tvb*, four different target sequences complementary to tvb^{s3} mRNA were tested. The DF-1 cell line is homozygous for tvb^{s3}, an allele that allows infection by ALV(B) and (D), but not (E) (Klucking et al., 2002). The various *tvb* shRNA-mir cassettes were delivered to DF-1 cells using the RCASBP(A) retroviral vector (transcribed from the proviral LTR). After viral titer peaked, *tvb* expression was assessed by Western blot using supernatant from cells expressing the SU(B) protein fused to a rabbit immunoglobulin tag (SU(B)–rIgG) as a primary antibody. Lysates from mock-transfected cells, cells transfected with a shRNA-mir containing a scrambled sequence, and quail QT6 cells that do not express *tvb* were included as controls. The four shRNA-mir sequences reduced *tvb* expression to varying degrees, with two of the targets (vari2 and cshl1) reducing expression significantly as compared with the scrambled shRNA-mir control (Fig. 3A). RT–PCR measurements demonstrated that *tvb* mRNA levels are also reduced by these two constructs, although not to the same extent as *tvb* protein levels (data not shown), consistent with the expected inhibition of translation in addition to an associated reduction in mRNA levels.

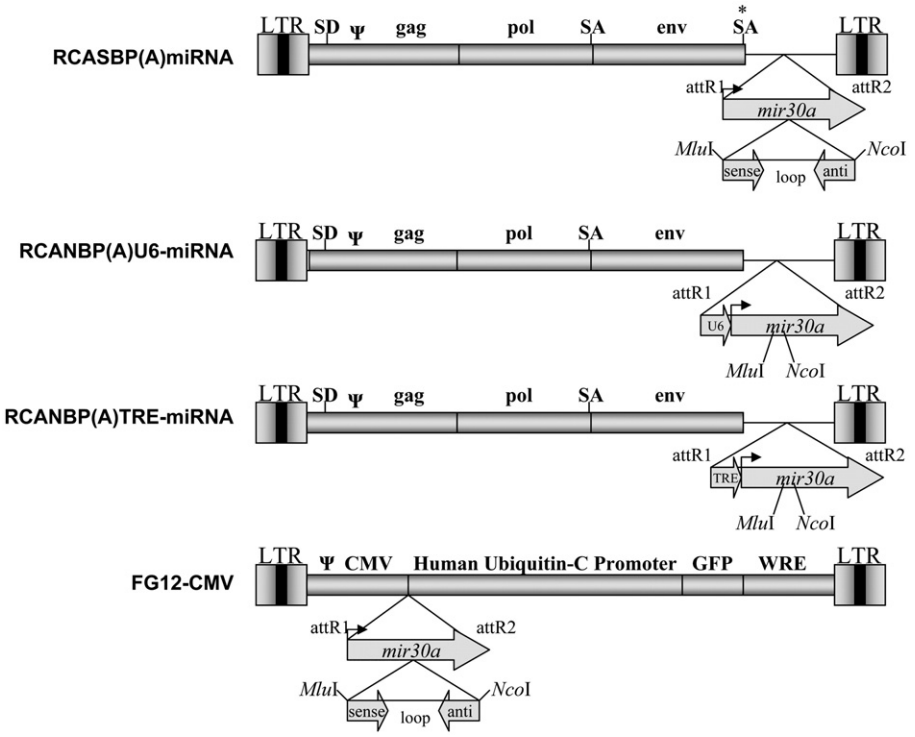

Fig. 1. Diagram of avian shRNA-mir vectors. Top line: shRNA-mirs inserted into the RCASBP(A) vector are expressed from the viral LTR via a spliced RNA transcript. Within the *mir-30a* gene, a short-hairpin loop containing the target sequence and its complement flanking the native *mir-30a* loop sequence is synthesized and inserted between the *Mlu*I and *Nco*I restriction sites. Second line: shRNA-mirs inserted into RCANBP(A) are meant to be transcribed from chicken U6 (or other pol III) promoters. Third line: shRNA-mirs inserted into RCANBP(A)TRE–miRNA are transcribed from a TRE (tetracycline-regulated) promoter (Holmen and Williams, 2005). Fourth line: shRNA-mirs inserted into the FG12-CMV defective lentiviral vector are transcribed from the CMV promoter. LTR – long terminal repeat; Ψ – packaging signal; SD – splice donor; SA – splice acceptor; attR – Gateway® recombination sites; WRE – Woodchuck Responsive Element.

To determine if the reduced *tvb* expression was sufficient to interfere with viral entry, a viral challenge assay was performed. DF-1 cells expressing the *tvb* shRNA-mir sequences were infected with RCASBP(B)AP virus, a form of ALV(B) that expresses an alkaline phosphatase (AP) reporter gene. RCASBP (B)AP infectivity was then assayed using a short-term AP titer assay, which measures AP gene expression from viruses that successfully gained entry into the cells. The level of interference to viral entry correlated with the level of reduction in *tvb* expression. Viral infectivity was significantly ($P<0.01$) reduced, about 2.5-fold by the cshl1 shRNA-mir and 2-fold by vari2, when compared to mock-transfected DF-1 cells (Fig. 3B). The other two targets and a control scrambled sequence target did not significantly reduce ALV(B) infectivity.

Expression of shRNA-mirs from pol II promoters allows for the use of various inducible promoters that can regulate the subsequent RNAi effect. Among other advantages, this provides a control that demonstrates that any resulting phenotype is dependent on the expression of the shRNA-mir and is not an unrelated response to the delivery vector. We transferred the shRNA-mir cassettes shown to be effective against *tvb* to the RCANBP(A)TRE–miRNA vector (Fig. 1) in which the shRNA-mir cassette is expressed from a synthetic promoter regulated by a tetracycline response element (TRE) that is inhibited in the presence of a tetracycline tet-off transactivating protein and doxycycline (Holmen and Williams, 2005). As shown in Fig. 4, a similar reduction in titer of the RCASBP(B)AP challenge virus was observed with these vectors as was seen when the shRNA-mirs were expressed from the viral LTR (Fig. 3B). However, in the presence of doxycycline and the tet-off regulatory protein, the RNAi effect was completely abolished (Fig. 4). This demonstrates the utility of the RCANBP(A)TRE–miRNA vector system and confirms that the specificity of the antiviral effect is due to the expression of the shRNA-mir cassette.

Pathogen-derived resistance through shRNA-mir silencing of env(B)

The ALV(B) surface glycoprotein (SU), encoded by *env(B)*, was targeted with five different shRNA-mir sequences (Materials and methods) predicted by two different algorithms. To determine which shRNA-mir reduced SU(B) expression most effectively, RCASBP(A)–shRNA-mir vectors containing these target sequences were propagated in DF-1 cells stably expressing SU(B)–rIgG. After the viral titers peaked, SU(B)–rIgG expression was assayed by Western blot (Fig. 5A). The 510 shRNA-mir reduced SU(B)–rIgG expression very effectively, followed by the 112 shRNA-mir. Surprisingly, the 854 shRNA-mir that was chosen by both algorithms showed no decrease in expression as compared with the scrambled sequence control.

To assess the corresponding effects on ALV(B) replication, we employed a defective lentiviral delivery vector with a GFP

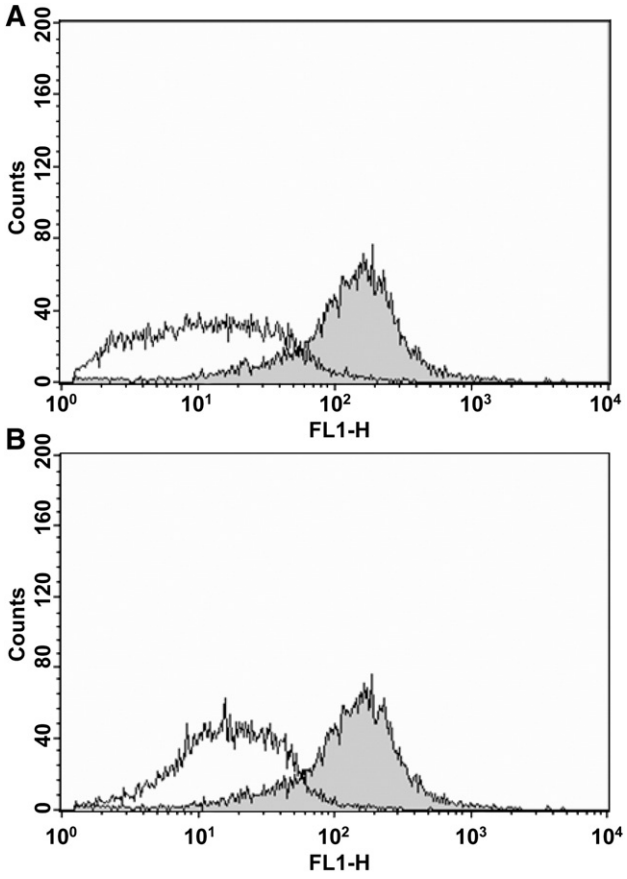

Fig. 2. RNAi vs. GFP using RCASBP(A)shRNA-mir. DF-1 cells stably expressing GFP were transfected with one of two RCASBP(A)shRNA-mir vectors containing sequences (Supplemental Table 1) directed against GFP and fluorescence was analyzed by FACS. The shaded area represents DF-1 cells transfected by a shRNA-mir with a scrambled control sequence while the unfilled curve represents cells transfected with the (A) GFP-1 shRNA-mir or (B) GFP-2 shRNA-mir.

reporter gene, *FG12-cmv* (Fig. 1), such that ALV challenge virus replication could be specifically monitored by ELISA for the ALV p27 capsid protein. The 510 shRNA-mir was inserted into the vector, to be transcribed from the viral cytomegalovirus (CMV) promoter. The defective virus was transfected into 293FT cells along with a plasmid that provides the vesicular stomatitis virus G protein (VSV-G) to provide a functional envelope protein. The VSV-G-pseudotyped delivery virus was then used to infect DF-1 cells. Multiple rounds of infection were performed to maximize the number of cells expressing the shRNA-mir. Analysis of GFP fluorescence indicated ~50% of the infected DF-1 cells were GFP positive. These cells then were infected with RCASBP(B)AP at a multiplicity of infection (MOI) of 0.01, 0.1, and 1.0. The cells were incubated in the presence of the challenge virus for 2 days, after which the media was replaced. ALV(B) propagation was assayed by ELISA. No significant difference was observed in viral spread between the 510 and scrambled shRNA-mirs at MOI values of 0.1 and 1.0 (data not shown). However, at an MOI of 0.01, viral replication was reduced. After 6 days, the level of p27 capsid protein detected in the cells expressing the 510 shRNA-mir was approximately half that detected in cells expressing the scrambled shRNA-mir and a third that of the mock-transfected cells (Fig. 5B).

Discussion

We have demonstrated the first use of retroviral vector-based RNAi against a viral pathogen in cultured chicken cells. Through the use of a shRNA-mir gene delivered via a replication competent retroviral vector, RCASBP(A), we successfully decreased expression of two genes necessary for ALV(B) replication, *tvb* and *env(B)*. This decreased expression resulted in reduced ability of ALV(B) to infect and propagate in DF-1 cells. In the case of the *env(B)* viral target, we were unable to use a replication-competent ALV(A) delivery vector because the vector alone reduced ALV(B) replication, presumably due to phenotypic mixing (Okazaki et al., 1975; Choppin and Compans, 1970). Thus, we resorted to a defective lentiviral vector that infected ~50% of the DF-1 cells. This probably contributes to the fact that we observed an antiviral effect only at low MOI, even though the 510 construct very substantially reduced *env(B)* expression when delivered by the RCASBP(A) vector. In addition, the shRNA-mir was expressed from the CMV promoter in the context of the lentiviral vector, and it is possible that this promoter is less efficient than the ALV LTR in

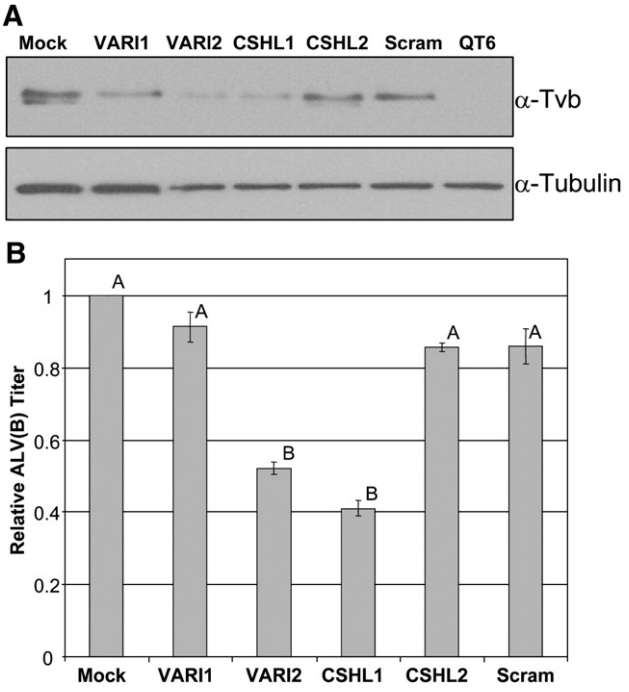

Fig. 3. RCASBP(A)shRNA-mir directed against *tvb* reduces protein expression and inhibits ALV(B) entry. Four different shRNA-mir sequences against tvb^{s3} (VARI1, VARI2, CSHL1, CSHL2; Supplemental Table 1), along with a scrambled control sequence, were delivered to DF-1 cells via the RCASBP(A) vector. (A) Western blot of *tvb* expression from cells infected with RCASBP(B) shRNA-mir vectors. Mock transfected cells and QT6 cells provide additional positive and negative controls, respectively. (B) RCASBP(B)AP titer on DF-1 cells infected with RCASBP(A)shRNA-mir vectors, relative to the titer on mock-infected DF-1. The MOI of the undiluted RCASBP(B)AP challenge virus was 4.0. Histogram bars labeled with different letters are significantly different ($P<0.05$).

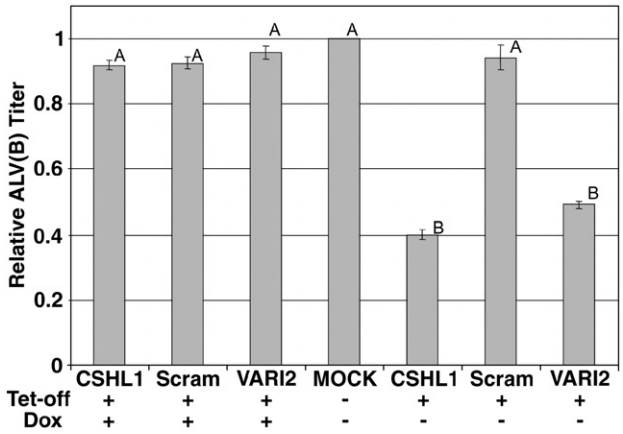

Fig. 4. Regulated promoter-driven inhibition of ALV(B). The miRNA sequences previously shown to be effective against *tvb*^{s3} (VARI2, CSHL1) were delivered to DF-1 cells via the RCANBP(A)TRE–miRNA vector (Fig. 1) that includes a regulated promoter whose activity is inhibited in the presence of doxycycline and the tet-off transactivating protein. These cells were challenged with ALV(B)-AP as in Fig. 3 both in the presence (+) or absence (−) of doxycycline (Dox). Histogram bars labeled with different letters are significantly different ($P<0.05$).

DF-1 cells. Moreover, Hu et al. (2002) found that siRNAs preferentially blocked late stages of viral replication (likely by being more effective against viral mRNAs as opposed to genomic RNA). This is consistent with our observation of no effect at high MOI, which mainly requires viral entry without additional spread, but a substantial effect at low MOI in which spread depends on multiple rounds of replication. Though increased shRNA-mir delivery or transcription efficiency may be required for increased resistance at higher MOIs, the low MOI experiment may be more representative of the viral concentrations that a live chicken would typically experience. In this regard, it is of note that even modest levels of receptor interference-based resistance to ALV *in vitro* provide substantial resistance to *in vivo* pathogenesis (Federspiel et al., 1991; Salter et al., 1998). By demonstrating the use of replication-competent, vector-based RNAi in chicken cells against an important viral pathogen, this study has opened the door for *in vivo* implementation. Anti-viral shRNA-mirs could be effectively delivered to chickens via transgenics (Salter et al., 1987; Mozdziak et al., 2003; McGrew et al., 2004) or as a part of a vaccine potentially to create viral-resistant chicken populations (Hu et al., 2002). The use of shRNA-mirs over siRNAs ensures the highest level of success by maximizing the level of gene silencing. Also, multiple shRNA-mirs can be delivered on a single transcript, so several different target sequences could be included to reduce the chance that the target virus will mutate and evade shRNA-mir silencing (Sun et al., 2006).

It should be noted that significantly greater levels of reduction in *tvb* and *env(B)* protein production (Figs. 3A and 4A) were observed than the 2- to 3-fold reductions in viral titers that result. Given that ALV(A) requires nearly non-detectable amounts of the *tvb* receptor to initially infect the cell, receptor protein expression may be a particularly challenging target for RNAi. In the case of *env(B)*, only ~50% of the cells appear to contain the defective lentiviral vector used to deliver the *env(B)* shRNA-mirs for ALV(B) challenge. Furthermore, as noted above, it's expected that the RNAi effect will be less at the stage of initial infection versus downstream viral replication. In these experiments, we chose *env(B)* as a target since our ALV(A) delivery vector shares homology in *gag* and *pol* with the ALV(B) challenge virus. Modifying the delivery virus and/or refining the targets, along with employing multiple targets, should allow one to achieve greater inhibition of viral titers.

We observed significant RNAi effects only when our shRNA-mirs were expressed from the viral LTR, TRE or CMV pol II promoters. Das et al. (2006) reported successful transcription of a chicken *mir-30a*-based shRNA-mir in DF-1 cells from a chicken U6 promoter, and Harpavat and Cepko (2006) describe an RCAS vector in which shRNAs are transcribed from a murine U6 promoter. In addition to the pol III promoters in these vectors, they also have, at least, an upstream LTR that can transcribe the RNAi insert, potentially giving rise to one or more transcripts that could be processed to siRNA. However, if this explains why the other vectors worked, it is then unclear why this effect was not sufficient in the pol III promoter vectors we examined. In several experiments using

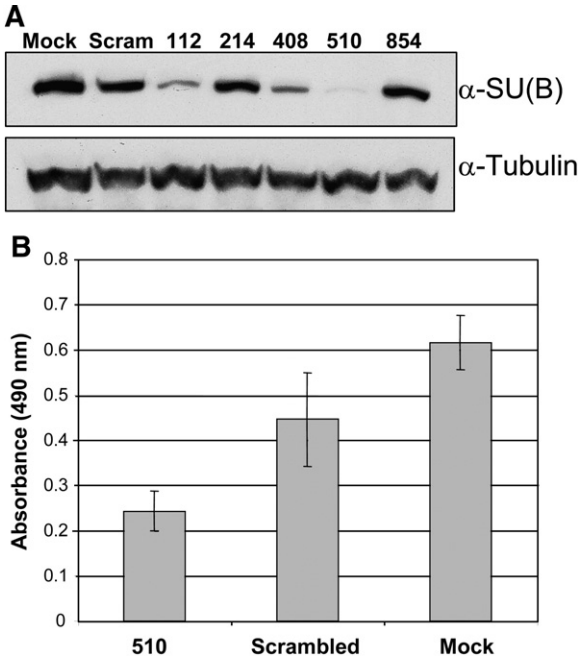

Fig. 5. RCASBP(A)shRNA-mir directed against *env(B)* reduces protein expression and limits ALV(B) infection. (A) Five different shRNA-mir sequences directed against *env(B)* (112, 214, 408, 510, 854; Supplemental Table 1) were transfected into DF-1 cells stably expressing SU(B)–rIgG, the surface glycoprotein encoded by *env(B)* fused to a rabbit immunoglobulin tag. After viral titer peaked, SU(B)–rIgG expression was assayed by western blot. Mock-transfected and scrambled shRNA-mir transfected SU(B)–rIgG cells are included as controls. (B) The 510 shRNA-mir was incorporated into the FG12-CMV lentiviral vector (Fig. 1) and delivered to DF-1 cells via VSV-G pseudotyping (Materials and methods). After multiple rounds of infection to maximize the fraction of infected cells, cells were challenged with RCASBP(B) at an MOI of 0.01, and viral spread was measured by ELISA for the ALV p27 capsid protein 6 days post infection.

other targets as either shRNA-mirs or shRNAs, we consistently failed to see an effect in chicken cells using any of a variety of both chicken and mammalian pol III promoters. The differences in vector performance likely relate to the sensitivity of the target systems under study and/or subtle differences in the vectors. Both Das et al. (2006) and Harpavat and Cepko (2006) used RCAS backbones for their pol III-driven cassettes, whereas we always used RCAN when employing a pol III promoter. In any case, transcription from the RNA polymerase II promoter opens the door for further regulation of shRNA-mir expression through tissue-specific or drug-inducible promoters. The use of a tet-regulated delivery vector allowed us to confirm that the antiviral effect we observed is specifically due to expression of the shRNA-mir cassettes employed (Fig. 4).

This study has demonstrated that retroviral RNAi may be a viable method for interfering with viral infection in chickens, while further developing an enhanced vector system for efficient gene silencing in avian cells. Further study is needed to demonstrate an antiviral effect *in vivo*, and the efficacy of implementing such an antiviral strategy, either through inclusion of the shRNA-mir in a vaccine or through creation of transgenic chicken lines, remains to be explored.

Materials and methods

Vector constructions

The chicken *mir-30a* gene is Accession No. MI0001204 at miRbase (http://microrna.sanger.ac.uk/) and is located at chr3:85,102,239-85,102,310 in build2 of the chicken genome sequence (http://genome.ucsc.edu/index.html?org=Chicken). This segment was amplified using an overlapping PCR technique (Ho et al., 1989) to insert *Mlu*I and *Nco*I restriction sites at the *mir-30a* target region. Primers used and the sequence of the *mir-30a* cassette are given in Supplemental Fig. 1. PCR was done in 100 μl containing 50 μl 2× PCR master mix (Promega Corp.), 100 ng of template, and primers at 1 μM each for 2 min at 95 °C followed by 30 cycles of 1 min at 94.5 °C, 1 min at 65 °C and 1 min at 72 °C. The modified chicken *mir-30a* cassette was inserted between *Bam*HI and *Not*I sites in the pENTR3C vector (Invitrogen Corp.), downstream of a pol III promoter (mU6, H1, cU6-1, cU6-2; Kudo and Sutou, 2005) with or without the 27 bp leader sequence normally present between U6 promoters and the start of U6 RNA (Paddison et al., 2004) or without any promoter. Two complementary (99 nt) oligonucleotides containing the desired target sequence with 5′ *Mlu*I and 3′ *Nco*I overhangs were synthesized (Invitrogen Corp.), annealed and ligated into the target region of the chicken *mir-30a*-derived entry vector. Specific target sequences for each gene were obtained using an in-house algorithm developed at the Van Andel Research Institute (VARI) by Matt VanBrocklin and Kyle Furge or by an on-line program from RNAi Central at the Cold Spring Harbor Laboratory (http://katahdin.cshl.org:9331/siRNA/RNAi.cgi?type=shRNA). Two shRNA-mirs predicted by each program for *tvb* (vari1, 2 and cshl1, 2, respectively) were tested. shRNA-mirs targeted against *env(B)* were named according to their first complementary nucleotide in the gene (Accession No. M14902). Three were obtained using the on-line Cold Spring Harbor Lab algorithm (214, 510, and 854), and three using the in-house Van Andel Research Institute algorithm (112, 408, 854), with the 854 target sequence selected by both algorithms. In total, 12 synthetic duplexes were employed with target sequences for GFP, *tvb*, *env(B)*, and a scrambled sequence was made as a negative control. Loop and flanking sequences are identical to the corresponding sequences of the chicken *mir-30a* gene. Sequences for all inserts are given in Supplemental Table 1. A Gateway© LR reaction was used to transfer the modified shRNA-mir gene into an appropriate Gateway-compatible destination vector (Holmen and Williams, 2005) according to the instructions from the manufacturer (Invitrogen Corp.). The shRNA-mir genes preceded by a pol III promoter were inserted into the RCANBP(A) destination vector and the *mir-30a* genes with no promoter were inserted into the RCASBP(A) destination vector. All entry vector inserts were confirmed by sequencing analysis, and retroviral vector constructs were verified by restriction enzyme mapping. The pEGFP-1 construct (Clontech, Palo Alto, CA) was digested with *Bam*HI and *Not*I, and the 0.7 kb GFP gene fragment was cloned into the *Bam*HI and *Not*I sites of pcDNA3 (Invitrogen Corp.) to create pcDNA3–EGFP.

A defective lentiviral vector, FG12-cmv, was kindly provided by Maria Soengas from the University of Michigan (Verhaegen et al., 2006). FG12-cmv was converted into a destination vector by digestion with *Hpa*I and ligation with Gateway conversion cassette C.1 (Invitrogen Corp.). Positive clones were selected by digestion with *Eco*RI and *Xba*I, and an LR reaction performed to insert the appropriate shRNA-mir cassette. Vectors employed for the tet-off analysis have been described previously (Holmen and Williams, 2005).

Cell culture

DF-1 and QT-6 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corp.) with 50 U/ml each of penicillin and streptomycin (Invitrogen Corp.), 10% fetal bovine serum (FBS, Hyclone), and 0.25 μg/ml of fungizone at 39 °C or in Leibowitz's L-15 and McCoy 5A media (1:1) supplemented with 10% FBS. 293FT cells were maintained in DMEM with 1% penicillin and streptomycin, 10 % FBS, and 1× MEM non-essential amino acid solution (Invitrogen Corp.) at 37 °C. DF-1 cells stably expressing GFP were selected in 500 μg/ml of G418 (Invitrogen Corp.) after transfection of the pcDNA3–EGFP plasmid. Clones were isolated using cloning cylinders (Bellco Glass Inc.), expanded, and maintained in standard medium supplemented with 500 μg/ml of G418. GFP expression was confirmed using a Becton Dickinson FACSCalibur (Lewis et al., 2001). GFP expression was also detected by fluorescence microscopy. DF-1 cells stably expressing SU(B) with a rabbit immunoglobulin tag, SU(B)–rIgG, were kindly provided by Mark Federspiel. SU(B)–rIgG expression was confirmed by Western blot analysis.

Virus propagation

Viral propagation was initiated by transfection of plasmid DNA that contained the retroviral vector in proviral form using calcium phosphate (Federspiel and Hughes, 1997) or SuperFect Transfection Reagent (Qiagen, Inc.) according to the manufacturer's protocol. In standard transfections, DF-1 cells were plated at 30% confluency, allowed to attach (2–3 h), and 5 μg of purified plasmid DNA was introduced by the calcium phosphate precipitation method previously described (Kingston et al., 1989), followed by a 5-min glycerol shock at 39 °C (15% glycerol in the medium). Viral spread was monitored by assaying culture supernatants for ALV capsid protein by ELISA (Smith et al., 1979). Virus stocks were generated from cell supernatants by centrifugation at 2000×*g* for 10 min at 4 °C and 0.45-μm filtration and were stored in aliquots at −80 °C. Lentiviruses were generated as previously described (Lois et al., 2002).

Virus infection

DF-1 cells were infected by incubation with diluted virus stock. Briefly, 5.0×10^5 cells were seeded on 10 cm dishes and allowed to attach (2–3 h) in 9 ml media. Serial dilutions of virus stock were produced and added to DF-1 media at MOIs of 1.0, 0.1, and 0.01. Cells were incubated in virus-containing media for 2 days, washed in PBS, and fresh media was replaced. Sample supernatants were subsequently collected every 2 days and infection monitored by ELISA as previously described (Smith et al., 1979).

For experiments involving doxycycline-regulated RNAi, 3.0×10^5 DF-1 cells per well were seeded on 6-well plates and allowed to attach (2–3 h) in 2 ml media. RCASBP(A)–tet-off (Holmen and Williams, 2005) and the appropriate RCANBP(A) TRE–miRNA viral construct (Fig. 4) were added at MOI = 1.0 in the presence of 4 μg/ml polybrene (Sigma). Coinfected DF-1 cells were passed to 10-cm dishes after 24 h, maintained in normal media in the presence or absence of 0.5 μg/ml doxycycline (Clontech), split 1:3 when confluent, and grown for 9 days before challenged with ALV(B).

ALV alkaline phosphatase (AP) challenge assay

DF-1 cells were seeded at a density of 2.5×10^5 cells per well of a 6-well dish. Serial dilutions of RCASBP(B)AP virus stock were produced and added to DF-1 media at MOIs of 4.0×10^0, 4.0×10^{-1}, 4.0×10^{-2}, 4.0×10^{-3}, 4.0×10^{-4}, 4.0×10^{-5}, and 4.0×10^{-6}. Cells were incubated with virus for 36–48 h at 39 °C. The alkaline phosphatase assay was modified from published procedures (Federspiel et al., 1994; Fekete and Cepko, 1993; Fields-Berry et al., 1992). Cells were fixed in 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) for 30 min at 25 °C, washed twice in PBS for 5 min each, and incubated for 1 h at 65 °C to inactivate endogenous AP activity. The cells were then washed twice with AP detection buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 min and exposed to the AP chromogenic substrates nitroblue tetrazolium (330 μg/ml) and 5-bromo-4-chloro-3-indoyl phosphate (170 μg/ml) (Roche). Enzymatically active AP produces an insoluble purple precipitate. The reaction was stopped by the addition of 20 mM EDTA, pH 8.0 in PBS. Purple cells were counted and the results were averaged to determine viral titer. The assay was performed a minimum of three times.

Statistical analysis

The ALV(B) titer data from four trials were analyzed with a mixed model where the RNAi treatment was treated as a fixed variable and the trials as random. The analyses were accomplished with SAS for Windows v9.1.3 (SAS Institute Inc., 2004). The differences of least squares means of the ALV (B) titers between RNAi treatments were pairwise tested for statistical significance. For convenience in visual evaluation, however, the relative average titer for each of the RNAi treatments was calculated by dividing each of the average titers with the average ALV(B) titer of the negative control, mock infection. The standard errors for the relative average ALV(B) titers were estimated from the variances and covariances corresponding to the average titer ratios between each of the RNAi treatments and the negative control following an approximate procedure as described by van Kempen and van Vliet (2000).

Polyacrylamide gel electrophoresis–Northern analysis (PAGE–Northern)

To quantify the amount of shRNA expression from the virus, total RNA was extracted from chronically infected cells using TRIzol reagent (Invitrogen Corp.). PAGE–Northern blot analysis was performed by separating 70 μg of total RNA on a 15% TBE-7M urea polyacrylamide gel (Bio-Rad Laboratories), and transferring to a nylon membrane (BrightStar-Plus, Ambion) by electro-transfer in 0.5× TBE at 200 mA for 30 min using a Western blotting apparatus (Hoefer). Blots were UV-crosslinked and subsequently probed with a 5′ end-labeled oligonucleotide that corresponds to the sense sequence of the shRNA used in the viral vectors. Blots were probed with 100 pmol of sense sequence, which was end-labeled with T4 polynucleotide kinase (Invitrogen) and [γ^{32}P]-ATP (125 μCi) (Amersham) and purified on a G-25 MicroSpin Column (Amersham). Probed membranes were washed and exposed to autoradiography film (Kodak).

Western blot analysis

Cell lysates were collected in 250 μl of 85 °C SDS loading buffer (50 mM Tris–HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The cell lysates were boiled for 10 min, vortexed vigorously and centrifuged for 10 min at 13,200×*g*. Alternatively, cells were lysed in 700 μl NP-40 lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl and 1% NP-40) supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF), centrifuged for 6 min at 13,200×*g* and boiled in

SDS loading buffer for 5 min. Proteins were electrophoresed on a 10% SDS–polyacrylamide gel for 2 h at 120 V and transferred to a nitrocellulose membrane for 1 h at 25 V. The membrane was blocked in 5% non-fat dry milk in TBS-T (25 mM Tris–HCl, pH 8.0, 125 mm NaCl, 0.1% Tween 80). To detect SU(B)–rIgG, the membrane was incubated with HRP-conjugated anti-rabbit IgG (Sigma) diluted 1:1000 in TBS-T for 1 h at room temperature. To detect *tvb*, membranes were incubated 1 h in 0.45-μm filtered media from SU(B)–rIgG expressing DF-1 cells, washed with TBS-T and incubated with HRP-conjugated anti-rabbit IgG diluted 1:1000 in TBS-T for 1 h at room temperature. Enzymatic chemiluminescence (ECL) substrate (Pierce Biotechnology) was added and the membrane developed on Kodak BioMax scientific imaging film (Kodak).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.04.013.

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